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Award Number: W81XWH-06-1-0323

TITLE: Checkpoint Functions of the BRCA1/BARD1 Tumor Suppressor

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REPORT DATE: July 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-07-2007 | Annual Summary | 1 Jul 2006 – 30 Jun 2007 |

**4. TITLE AND SUBTITLE**

Checkpoint Functions of the BRCA1/BARD1 Tumor Suppressor

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-06-1-0323

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Columbia University
New York, NY 10032

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
The breast and ovarian-specific tumor suppressor BRCA1 has been implicated in numerous cellular processes, including DNA repair, cell cycle checkpoint control, and mitotic spindle assembly. *In vivo*, BRCA1 primarily exists in association with BARD1 and the BRCA1/BARD1 heterodimer is thought to mediate the tumor suppression activity of BRCA1. It has been previously shown that the phosphorylation state of the BARD1 polypeptide is cell cycle regulated and that BARD1 is hyperphosphorylated in mitosis at seven distinct residues. To study the function of mitotic BARD1 phosphorylation, I used an siRNA-mediated approach to knockdown endogenous BARD1 expression and then restore expression with siRNA-resistant exogenous forms of BARD1. In this manner, I will evaluate the role of BARD1 mitotic phosphorylation in the G2 accumulation checkpoint, decatenation checkpoint, and homology-directed DNA repair. My initial studies showed that siRNA-mediated knockdown of BARD1 leads to a considerable IR-induced G2 accumulation checkpoint defect, illustrated by an ~8-fold increase in the percent of cells that enter mitosis following IR damage relative to control cells. Reconstitution experiments with mRNAs resistant to knockdown by BARD1-specific siRNAs resulted in ~ 3-fold decrease in the percentage of cells that escape the IR-induced G2 accumulation checkpoint. We are currently testing the role of individual phosphorylation sites in the checkpoint by reconstituting BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing mutations of specific phosphorylation sites.

**15. SUBJECT TERMS**
BARD1; BRCA1; siRNA; DNA damage, checkpoints

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 6 | 19b. TELEPHONE NUMBER *(include area code)* |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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**Introduction**: The objective of my research is to determine the role of BARD1 phosphorylation in the checkpoint functions of the BRCA1/BARD1 heterodimer [1]. In my original application, I proposed to achieve this by generating and characterizing isogenic subclones of HCT116 cells that express different knock-in alleles of BARD1. In the past year, however, I also tested the feasibility of an alternative approach based siRNA-mediated depletion of endogenous BARD1 coupled to transient reconstitution with exogenous BARD1. This approach has several clear advantages over the original knock-in strategy. First, since it involves transient transfection of a cell population, this approach is not susceptible to artifacts that arise due to clonal variation. Second, unlike the knock-in strategy, which is restricted to certain pseudo-diploid cell lines such as HCT116, this approach can be applied to a broad range of cell types. Third, this approach is more facile since it does not require the laborious process of generating stable knock-in subclones by targeted gene recombination.

**Body:** To implement this approach, I first designed two distinct siRNAs (siRNAs A and B) that can greatly reduce endogenous BARD1 expression (>90%) in a variety of cell lines. Second, by site-directed mutagenesis I introduced non-coding mutations into our BARD1 mammalian expression plasmids that render the resultant mRNAs resistant to knockdown by either siRNA A or B. With these reagents, we should be able to test whether BARD1 phosphorylation is required for specific checkpoint functions of BRCA1. For example, a BRCA1-dependent function, such as the IR-induced $G_2$ accumulation checkpoint, should be ablated by siRNA-mediated BARD1 knockdown, either as a direct consequence of BARD1 inactivation or as an indirect consequence of BRCA1 instability in the absence of BARD1. In either case, transfection of the siRNA-treated cells with a siRNA-resistant vector encoding wildtype BARD1 should rescue the checkpoint. If, however, a specific phosphorylation site (for example, S251) is required for the $G_2$ accumulation checkpoint, then transfection with a siRNA-resistant vector encoding S251A-mutant BARD1 should restore the expression levels of BRCA1 but not rescue checkpoint activity. Thus, by reconstituting siRNA-treated cells with siRNA-resistant expression vectors encoding the full panel of wildtype and phosphorylation site mutant BARD1 polypeptides, we should be able to identify the precise requirements for BARD1 phosphorylation in checkpoint function. A similar strategy was used successfully by Yu *et al.* to demonstrate a requirement for BACH1 phosphorylation in the same IR-induced $G_2$ accumulation checkpoint [2]; thus, we will be able to use BACH1 siRNAs and siRNA-resistant BACH1 expression plasmids kindly provided by Dr. Junjie Chen as reliable controls for analysis of the $G_2$ accumulation checkpoint [2]. Moreover, this strategy can be used not only to study the $G_2$ accumulation checkpoint (as proposed in Task 1), but also the IR-induced mitotic exit checkpoint (Task 2) and other BRCA1-dependent functions, such as the decatenation checkpoint and HDR-mediated double-strand DNA break repair.

**Key Research Accomplishments:** Two different BARD1-specific siRNAs (A and B) were designed to have a GC content of 30-50% and a general sequence of AA(19N)TT targeting the coding region of BARD1 mRNA. The chemically synthesized RNA duplexes were also created to have 3' overhanging UU dinucleotides, since these are reported to be most effective in knocking down the intended target [3]. The target sequence of siRNA A lies in coding exons 2/3 of BARD1, while that of siRNA B resides in coding exon 9. siRNA-mediated knockdowns were conducted by transient transfection of 293 cells with HiPerfect (Qiagen), a reagent that allows for highly effective gene silencing at low siRNA concentrations that minimize off-target effects. In pilot experiments, we observed that two sequential siRNA transfections (8 nM), approximately 24 hours apart, yields a highly effective knockdown (>90%) of BARD1 with either the A or B siRNAs, as compared to cells treated with the non-targeting (negative control) siRNA. The knockdown efficiencies were monitored at both 48 hrs and 72 hrs post-second transfection by Western analysis.

**Reportable Outcomes:** In evaluating the effect of BARD1 knockdown on the G2 accumulation checkpoint, siRNA-mediated knockdown of BRCA1 was included as a positive control, since BRCA1 is known to be required for this checkpoint [2]. Approximately 48 hrs and 72 hrs post-second transfection, one set of cells was irradiated with 10 Gy, while a second set was mock treated. After three hours at 37$^{o}$C, both treated and mock-treated cells were incubated for 15 hours with nocodazole (1ug/mL) to arrest cells in mitosis. The cells were then fixed with 70% ethanol and placed at -20$^{o}$C overnight. The mitotic population of each culture was then measured by flow cytometric analysis after staining with propidium iodide and the mitotic marker, phospho-histone H3. As expected, knockdown of BRCA1 caused a defect in activation of the G2 accumulation checkpoint. Significantly, BARD1 knockdown also induced a checkpoint defect, as illustrated by an ~8-fold increase in the percentage of cells that entered mitosis following IR treatment relative to control cells.

To confirm that the observed checkpoint defect is due to BARD1 knockdown, and not to non-specific off-target effects of the siRNAs, we introduced silent mutations into the siRNA-specific targeting regions of a BARD1 mammalian expression vector to render its mRNA product resistant to either the BARD1-specific siRNA A or B. Two mutations, especially if placed together near the middle of the siRNA sequence, are generally sufficient to ablate siRNA-mediated knockdown, although more mutations can only help [3]. In our design of siRNA-resistant BARD1 expression vectors, we were able to introduce 3 or 4 tandem nucleotide changes that disrupted siRNA complementarity but did not alter the coding potential of the vector. Of note, the BARD1 polypeptides encoded by these vectors contain an N-terminal tag of three tandem FLAG epitopes that allows the endogenous and exogenous (i.e., vector-encoded) forms of BARD1 to be distinguished in rescue experiments. To ascertain whether the G2 accumulation checkpoint of the BARD1 siRNA-treated cells is due to BARD1

depletion, 293 cells that had been BARD1-depleted by two successive siRNA transfections (with siRNAs A or B) were transiently co-transfected with the appropriate siRNA-resistant BARD1 expression vector. Western blot analysis with a FLAG-specific antibody confirmed successful expression of exogenous BARD1 in siRNA-treated cells. Significantly, these cells displayed an ~ 3-fold decrease in the percentage of mitotic cells after IR exposure, indicating that reconstitution of BARD1-depleted cells with siRNA-resistant wildtype BARD1 provides a partial rescue of the G2 accumulation checkpoint.

The results described here were recently reported as an abstract at the Annual Meeting of the American Association of Cancer Research in April, 2007 [4].

**Conclusion:** With this system of BARD1 rescue in place, we are now in a position to test the role of individual BARD1 phosphorylation sites in this checkpoint by reconstituting BARD1-depleted cells with siRNA-resistant BARD1 polypeptides bearing defined mutations of specific phosphorylation sites. This approach will be used in the coming year to determine whether specific BARD1 phosphorylation events are required for BRCA1-dependent processes, such as the G2 accumulation checkpoint.

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